IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor(s):	**Cox et al**		
Serial No:	**10/081,771**	Examiner:	**H. Calamita**
Filed:	**20 February 2002**	Art Unit:	**1637**
For:	**MISMATCH REPAIR DETECTION**	Confirmation No:	**4176**

Declaration by Dr. Stephen B. del Cardayre Under 37 C.F.R. 1.132

Commissioner for Patents
P.O. Box 1450
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Sir:

I, Stephen B. del Cardayre, declare as follows:

1. I received a Bachelore of Arts degree in Biochemistry in 1987 from the University of California, Berkeley, and a Doctor of Philosophy degree in Biochemistry in 1994 from the University of Wisconsin-Madison. From 1994-1997, I was a Post-doctoral Fellow in the laboratory of Dr. Julian E. Davies, in the Department of Microbiology at the University of British Columbia. During my postdoctoral fellowship, I studied the microbiology and molecular genetics of Gram-positive bacteria, with emphasis on mechanisms of pathogensis and drug resistance, and the development of antibacterial agents. From 1997-2002, I was a Staff Scientist at Maxygen, Inc., where I worked on the development and application of directed evolution technologies at the levels of individual enzymes, metabolic pathways, and whole organism to solve problems in the chemicals industry. During this time, I was Principal Investigator of a NIST-ATP grant todevelop whole genome shuffling technologies, such that the proprietary Maxygen technology could be expanded from single genes to whole genomes. From 2002-2003, I was Science and Technology Officer, Codexis, Inc. where I directed research for new technology development, led external collaborative research programs, and led the genome shuffling program. From 2004 until the present, I have been Head of Microbiology at Codexis, Inc., having responsibility for the management of the microbiology group and for internal

and collaborative product and technology development programs. Among other professional activities, I have been a Panel Review Member of the Interagency Initiative for Metabolic Engineering, National Science Foundation, in 1999 and 2001; I was an External Reviewer, U.S. Department of Agriculture-National Research Initiative Competitive Grants Program, Value-Added (Non-Food) Products and Bio-Fuels Research Program (1995-present); and I serve on the Board of Directors of the Society for Industrial Microbiology (2004-present). As indicated by the list of publications and presentations attached as Exhibit A, I am an expert in the field of microbial genetics and have particular interests in the use of directed evolution to develop bacterial stains having significant industrial and medical applications.

2. I am neither an employee of nor do I have a direct financial interest in either the University of California, assignee of the above application, or ParAllele Bioscience, Inc., exclusive licensee of the above application.

3. I fully understand the invention described in the above application, and in particular, the role of methyl-directed mismatch repair systems in the detection scheme of the invention. I have also read and understand the following references relevant to the invention:

Fang et al, "Methyl-directed repair of mismatched small heterologous sequences in cell extracts from *Escherichia coli*," J. Biol. Chem., 272: 22714-22720 (1997).

Carraway et al, "Repair of heteroduplex DNA molecules with multibase loops in *Escherichia coli*," J. Bacteriol., 175: 3972-3980 (1993).

Parker et al, "Repair of DNA heteroduplexes containing small heterologous sequences in *Escherichia coli*, " Proc. Natl. Acad. Sci., 89: 1730-1734 (1992).

Below, I discuss genetic variability and manipulation of bacteria in general, and more particularly, I address whether the *in vitro* evidence presented in Fang et al (cited above) would suggest that bacteria having similar properties *in vivo* could be readily obtained.

Background

4. Evolution is the iterative process of genetic diversification and natural selection that is believed to be the process by which all biological diversity has come to be. By controlling in the lab the processes of genetic diversification and functional selection, the evolutionary algorithm can be applied to direct the evolution of biological systems to have desired functional traits. "Directed evolution" is arguably the most robust approach to purposely engineer biological function (Tobin, Gustafsson et al. 2000; del Cardayre and Powell 2003) and has been successfully used to engineer biological systems to have new activities, many which have no known natural precedent.

5. The applications of directed evolution are diverse, and today the technology is being applied throughout the biotechnology industry. Targeted systems range from single genes and proteins to complete metabolic pathways and organisms. Improvements include increased "activity" (chemical conversion (Castle, Siehl et al. 2004), binding (Lazetic, Leong et al. 2002), expression (Crameri, Whitehorn et al. 1996), etc) broadened or new selectivity (Zhang, Dawes et al. 1997; Raillard, Krebber et al. 2001), and adaptation to new environmental conditions (Ness, Welch et al. 1999). The changes in activities that can be achieved are dramatic and often are measured in orders of magnitude (Crameri, Raillard et al. 1998; Castle, Siehl et al. 2004). The most common published examples of the application of directed evolution are for the alteration of the *in vitro* and *in vivo* activities of enzymes (Powell, Ramer et al. 2001), but they also include the improvement of proteins (Chang, Chen et al. 1999; Punnonen 2000; Whalen, Kaiwar et al. 2001), operons and metabolic pathways (Crameri, Dawes et al. 1997; Newman, Garcia et al. 2004), plasmids, viral and microbial genomes (Demain and Solomon 1986; Patnaik, Louie et al. 2002; Zhang, Perry et al. 2002). Since all biological systems were created through the evolutionary process, all biological systems are theoretically amenable to "improvement" through directed evolution. An important finding is that activities that have never been observed in nature can be created using directed evolution (Raillard, Krebber et al. 2001).

6. The two main components of directed evolution are genetic diversification and functional screening. The DNA encoding the system to be improved is identified. This could be a gene encoding an enzyme of interest or a whole genome of useful microorganism. This DNA is then "diversified," which involves the introduction of mutations or changes in DNA sequence such that a population of new DNAs are created that are similar but different to the starting sequence and each other. This "library" of new DNAs is then screened for those unique individuals that have acquired the desired new activity. There are many methods for effecting the diversification of DNAs ranging from the introduction of random mutations by error prone PCR or chemical mutagenesis, the introduction of specific mutations through the use of oligonucleotide directed mutagenesis, and the recombination or shuffling of populations of genes that are known to contain useful mutations.

7. Once a library has been created it is "screened" for those members that have attained an improvement in the desired activity. This requires expressing the DNA library under conditions where the desired activity can be detected and quantified from individual library members. Those members that demonstrate improvements in the desired activity are "selected" for further genetic diversification and screening. This recursive process is continued until the desired activity is reached.

8. The literature is rich with examples of the successful application of directed evolution. Early examples include the improvement of industrial strains of bacteria and fungi for increased fermentation productivity (Demain and Solomon 1986) or an increase in tolerance to industrial stress tolerance, such as elevated temperature, solute concentration, or pH extremes. These phenotypic changes can be achieved through the combination of mutation and selective pressure. Modern tools of molecular biology now allow the improvement of whole cells through the improvement of specific targeted genes, for example those known to be involved or limiting a particular cellular activity. The targeted DNA is diversified, and the library screened for individuals able to confer upon the cell the desired phenotypic change. For example, Stutzman et al recently evolved the ability of *Streptomces avermitilis* to produce predominantly avermectin B1 as opposed to a 1:1 mixture of avermectin B1 and B2 by directing the evolution of the *ave*C gene (Stutzman-Engwall, Conlon et al. 2003; Stutzman-Engwall, Conlon et al. 2005). Similarly, Castle et al created a new gene that confered upon plants glyphosate restance (Castle, Siehl et al. 2004). This required identifying a gene from a bacterium that had extremely low glyphosate acetyltransferase activity, improving its catalytic activity ~ 10,000-fold, and then expressing it in plants. In this program, the team produced the enzyme variants in the bacterium *Echerischia coli*. They then measured the enzyme activity in an *in vitro* high-throughput enzymatic assay (the screen). Genes encoding the improved enzymes were then expressed in plants, and the improved activity was expressed *in vivo* in the plant, providing high level glyphosate resistance. This example illustrates two important points. First, activities that are detectable can be dramatically improved. Second, that activities detected and improved *in vitro* can translate to improved activity *in vivo*. Numerous similar examples exist in the public and private domain.

9. Directed evolution is not magic. It is a gradual process that allows small but significant changes in phenotype to be achieved in each round of diversification and selection. More dramatic changes can be realized after several evolutionary rounds. If an activity can be detected, it usually can be significantly improved. Industrial antibiotic fermentations are a good example. The natural isolate of a producing organism usually makes ~100 mg/L of antibiotic, while mature strains that have been through many rounds of directed evolution often produce >>10g/L. It is unlikely you would achieve 100-1000-fold improvement in a single round or from a single mutation (although it can happen). In the case of enzymes, it is difficult to predict the level of improvement. There are examples where enzymes that had no detectable activity on a particular substrate were evolved to have that activity in a single round of evolution (Raillard, Krebber et al. 2001). While there are other cases where many rounds of evolution are required to achieve significant improvements. In general, if there is detectable activity, and the new or improved activity requires similar molecular interactions, significant improvements 2-10 fold should be readily achievable in several rounds of evolution.

Modification of a Methyl-Directed

Mismatch Repair System

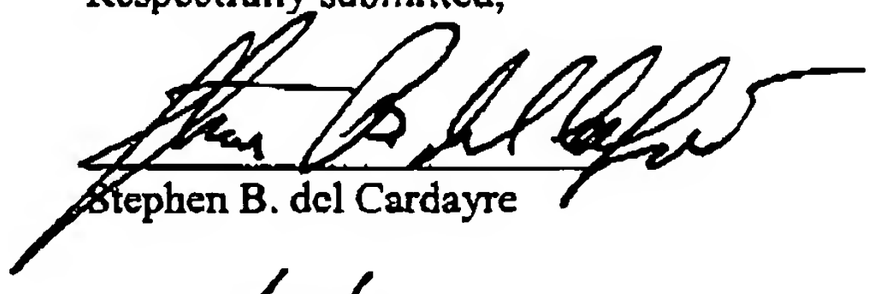
10. The methyl directed mismatch repair (MDMR) system of *E. coli* (and other organisms) is amenable to directed evolution. The system demonstrates a broad spectrum of DNA structures that it is capable if repairing (Parker and Marinus 1992; Fang, Wu et al. 1997). Further, the breadth of activity appears to be subject to the conditions under which repair is measured, and the structure of the MDMR components (Scharer, O.D., 2003). The *E. coli* MDMR has been observed to correct insertions of up to 4bp *in vivo* (Parker and Marinus 1992). Yet, it has been observed to repair insertions of more than 7bp when measured *in vitro* (Fang, Wu et al. 1997). Since the MDMR system already has the ability at a molecular level to correct insertions/deletions arguably > 7 bp, this activity needs to be improved and adapted to function in the intracellular environment of *E.coli* to reach the same level of activity as observed for 1-4 bp insertion/deletions *in vivo*. This increased expression of broader specificity *in vivo* could be achieved in at least two ways. First, the system could acquire mutations that would improve the molecular interactions between the MDMR components and the larger loop substrates such that repair of such substrates would improve both *in vitro* and *in vivo* i.e the activity becomes so high *in vitro* that it is observable *in vivo* even though the ratio of the *in vitro* and *in vivo* activity levels would remain the same. Alternatively, the MDMR components could acquire mutations that allow the *in vitro* observed activity toward large loop structures to be expressed in the new environment of the *E. coli* cytoplasm. This latter class of changes is perhaps the most readily observed in directed evolution research, and they represent the adaptation of biological activities to new environments. Improved expression of foreign genes in heterologous hosts (Crameri, Whitehorn et al. 1996) and improved enzyme activity in extreme environments (Moore and Arnold 1996; Ness, Welch et al. 1999) are examples of this type of evolution. If a directed evolution program were targeted to the components of the MDMR system, *mutH,L, and S*, and the resulting libraries were expressed and screened *in vivo*. An increase in the ability to repair substrates containing >> 4 bp insertions/deletions would likely arise through both of the mechanisms described above.

11. The expansion of the mismatch range of the MDMR system could be achieved by either mutating the entire organism and selecting for mutants that have acquired the ability to correct mismatches containing larger loop structures. Alternatively, *mut*H,L, and/or *mut*S could be diversified with the resulting libraries being screened *in vivo* or *in vitro* for variants supporting the desired expansion of activity.

12. Since *E. coli* MutS already interacts productively with 3 and 4 bp loops (Parker and Marinus 1992), and homologs recognize both smaller and larger loops (Scharer 2003), MutS represents a functionally diverse protein family, and there is no obvious molecular reason why it could not be evolved to recognize loops of significantly larger size. There are examples of the evolution of restriction endoncleases to recognize 8 bp as opposed to 6 bp sequences (Lanio, Jeltsch et al. 1998). Further a single amino acid change in the active site of RNase A converts the enzyme from an endonuclease to a processive exonuclease when catalyzing the hydrolysis of polyA (delCardayre and Raines 1994). Thus, this type of protein nucleic interaction can be altered. Since dramatic expansions in substrate specificities and increases in catalytic activities have been observed in many directed evolution programs, it would be difficult to put a limit on the size of loop that the MDMR system could be evolved to repair. Biological systems are remarkably adaptable. When put to the test, "life finds a way."

13. I further declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,



Stephen B. del Cardayre

3/21/05

Date

Attachment: Exhibit A

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scale production of Doramectin by *Streptomyces avermitilis*." Metab Eng, 2005, Jan; 7(1): 27-37.

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Exhibit A

Stephen B. del Cardayre

List of Lectures and Publications

Selected Invited lectures:

"Evolutionary Engiineering of Industrial Biocatalysts through gene, pathway and genome shuffling." Society for Industrial Microbiology annual meeting, Anaheim, CA, November, 2004.

"Getting What We Screen For: Industrial Biocatalysts through gene, pathway, and genome shuffling." BioCat2002, Philidelphia, PA, April 2002.

"Genome Shuffling of Industrial Microorganisms." Society for Industrial Microbiology, St. Louis, MO, 2001.

"Genome Shuffling: Accelerating Commercial Strain Improvement" International Symposium for the Biology of Actinomycetes. Vancouver, B.C.. 2001.

"Directed Evolution of New Biocatalysts by DNA Shuffling," 50th Meeting of the Society for Industrial Microbiology, Alexandria, VA, 1999.

"Improving the Function and Production of Natural Products through DNA Shuffling," Meeting of the American Association of Pharmaceutical Scientists, San Francisco, CA, 1998.

"DNA Shuffling of Gene, Proteins, and Pathways," Pacific Northwest Symposium for the Biology of Streptomyces, Mt. Rainier, Washington, 1997.

Publications:

1. del Cardyare, S.B. "Developments in Strain improvement Technologiy: Evolutionary Engineering of Industrial Microorganisms through Gene, Pathway, and Genome Shuffling." In *Natural Products: Drug Discovery and Therapeutic Medicine*, Humana Press, in press.

2. Koffas, M. and del Cardayre, S. "Evolutionary Metabolic Engineering." *Metab Eng.* 2005 Jan;7(1):1-3.

3. del Cardayre, S. and Powell, K. "DNA Shuffling for Whole Cell Engineering" in Handbook of Industrial Cell Culture: Mammalian, Microbial, and Plant Cells, Humana Press. 2003.

4. Patnaik, R., Louie, S., Gavrilovic, V., Perry, K., Stemmer, W.P., Ryan, C.M., del Cardayre, S. "Genome shuffling of Lactobacillus for improved acid tolerance."*Nat Biotechnol.* 2002 Jul;20(7):707-12.

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6. K.A. Powell, S.W. Ramer, S.B. del Cardayre, W.P.C. Stemmer, M.B. Tobin, P.F. Longchamp, G.W. Huisman. 2001. "Directed Evolution and Biocatalysis." *Angew. Chem. Int. Ed.*, 40, 3948-2959

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12. M. Ribo, S. B. delCardayré, R.T. Raines, R. De Llorens , and C.M. Cuchillo. 1996. "Production of Human Pancreatic Ribonuclease in *Sacharomyces cerevisiae* and *Escherichia coli*." *Protein Expression and Purification*, 7(3):253-261.

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16. S. B. delCardayré. 1994. Catalysis by Ribonuclease A: specificity, processivity and mechanism. PhD thesis, University of Wisconsin-Madison, Department of Biochemistry.

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20. S. B. delCardayré and J. B. Neilands. 1990 "Structure- Activity Corellations for the Ferric Uptake Regulation (FUR) Repressor Protein of *E. coli* K12." in *Iron Biominerals*, Plenum Press, New York, pp 389-396.

Patents:

Issued U.S. Patents

Document No.	Date	Title
6,716,631	2004-04-06	Evolution of whole cells and organisms by recursive sequence recombination.
6,686,515	2004-02-03	Homologous recombination in plants.
6,528,311	2003-03-04	Evolution of whole cells and organisms by recursive sequence recombination.
6,352,859	2002-03-04	Evolution of whole cells and organisms by recursive sequence recombination.
6,335,198	2002-01-01	Evolution of whole cells and organisms by recursive sequence recombination.
6,326,204	2001-12-04	Evolution of whole cells and organisms by recursive sequence recombination.
6,287,862	2001-09-11	Evolution of whole cells and organisms by recursive sequence recombination.
6,107,068	2000-08-22	Coenzyme A disulfide reductase, and inhibitors thereof useful as antimicrobial agents.

Published U.S. Applications

Document No.	Date	Title
2002/0137153	2002-09-26	Enantioselective production of amino carboxylic acids.
2002/0072097	2002-06-13	Molecular breeding of transposable elements.

Published International Applications

Document No.	Date	Title
WO 02/04629	2002-01-17	Molecular breeding of transposable elements.

WO 01/38504	2001-05-31	Homologous recombination in plants.
WO 01/12817	2001-02-22	Evolution and use of enzymes for combinatorial and medicinal chemistry.
WO 01/00234	2001-01-04	Methods and compositions for engineering of attenuated vaccines.
WO 98/31837	1998-07-23	Evolution of whole cells and organisms by recursive sequence recombination.
WO 97/23628	1997-07-03	Staphylococcus aureus coenzyme A disulfide reductase, and inhibitors thereof useful as antimicrobial agents.